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The effects of retinoic acid (RA) in breast cancer have been widely studied. Treatment with RA leads to either growth arrest or apoptosis in breast cancer cells. The cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ may be involved in terminal differentiation associated growth arrest. We showed in this study that PML/RARα increased the transcription of *p21$^{WAF1/CIP1}$* gene and the activation was further induced by RA treatment. Deletion analysis revealed a region upstream of the *p21$^{WAF1/CIP1}$* promoter that is required for transactivation by PML/RARα. Transient transfection of PML/RARα in cells increased the endogenous p21$^{WAF1/CIP1}$ protein levels. These results suggest that the effects of RA may be a result of the activation of *p21$^{WAF1/CIP1}$* by PML/RARα.

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Introduction

Breast cancer is one of the most prevalent malignancies in women and accounts for the highest morbidity among women suffering from cancers (1). The oncogenic development of breast cancer is accompanied by genetic alterations of multiple oncogenes, tumor suppressor genes, and other factors. Overexpression of the epidermal growth factor receptor (EGFR), HER-2/neu, and myc oncogenes are some of the well described genetic changes that frequently occur in breast cancer (2-4). In addition, deletions of chromosomal loci that are thought to be associated with putative tumor suppressors including the p53, BRCA1 and BRCA2 genes, also contribute to a more aggressive phenotype of breast cancer (5). These genetic changes have important prognostic implication in the clinical outcome of breast cancer.

Epidermal growth factor (EGF) is a small polypeptide that stimulates cell proliferation in both cell culture and in intact animals (6). EGF has been shown to promote both normal and neoplastic growth of mammary tissue in rodents (7) and in human breast cancer cells in culture (8-10). The biological effects of EGF are mediated through high affinity binding to EGFR, which is a 170 kDa membrane receptor tyrosine kinase (6). There is great interest in the study of EGFR in human breast cancer, however, the clinical relationships and prognostic value of the receptor in breast cancer are still unclear (11).

The transforming growth factor-α (TGFα), which bears considerable sequence homology to EGF and is produced by many transformed cells, also binds to EGFR and mimics the action of EGF (6,12). Expression of EGFR in breast cancer cells is regulated by mitogenic growth factors, and also by the superfamily of nuclear hormone receptors, which includes estrogen, progesterone, glucocorticoids, and retinoic acid receptors (13). Therefore, optimal regulation of EGFR expression is a complex process involving the coordinate interaction of several heterologous growth factors and hormones, whereby the proliferation of normal and neoplastic breast cells can be modulated. Although the clinical significance and prognostic value of EGFR in human breast cancer are unresolved, the involvement of EGFR in the growth of normal and malignant human mammary cells indicates that it may play a critical role in the oncogenesis of human breast carcinomas. Further evidence of the importance of EGFR in the development of breast cancer has been shown in transgenic mice studies where the overexpression of TGFα causes a significant increase in the occurrence of mammary carcinomas (14-16).

Specific chromosomal abnormalities occur frequently in acute and chronic leukemias (17). These cytogenetic aberrations are thought to contribute to leukemogenesis. Furthermore, significant differences in the type of genes involved in chromosomal translocations in acute leukemias and chronic leukemias have also been observed. For example, study of the recombination of bcr and abl genes in the t(9;22) of CML, and the myc and immunoglobulin genes in the t(8;14) of Burkitt lymphoma, has led to the identification of new fusion genes involved in the neoplastic transformation of these hematopoietic tumors (17-19). It has also been shown that a chromosomal translocation breakpoint t(15;17) occurs in over 90% of all patients with acute promyelocytic leukemia (APL), a subtype of acute myeloblastic leukemia (20-23). The recombination involves the PML (myl) gene on chromosome 15 and the retinoic acid receptor-α (RARα) on chromosome 17. The chimera PML/RARα and RARα/PML genes are formed as a result of the reciprocal translocation between the PML and RARα loci (20-22, 24). The PML/RARα cDNA has been isolated and shown to encode a fusion protein that is retinoic acid responsive and exhibits transactivation potential in a cell type- and promoter-specific manner differing from the wild-type RARα (25-27). Since the administration of all-trans retinoic acid to APL patients leads to rapid achievement of remission, it has been suggested that disruption of RARα may be part of the underlying pathogenesis of APL (28-30). The biological function and etiologic implications for the PML gene is not known and leaves open the question of its role in APL. Characterization of PML reveals that it is a putative zinc finger protein and transcription factor that shares homology with a newly recognized family of proteins that includes a variety of putative transcription factors as well as the recombination-activating gene product (RAG-1) (25-

27,31). Expression of *PML* is found in a variety of fetal and adult tissues including brain, gut, liver, lung, muscle, placenta, and testes (31,32).

Our laboratory, in collaboration with Dr. Kun-Sang Chang at the University of Texas, M.D. Anderson Cancer Center, investigated the role of t(15;17) chromosomal translocation in the leukemogenesis and the emergence of multidrug resistance in acute promyelocytic leukemia (APL). We demonstrated that *PML* suppresses the clonogenicity and tumorigenicity of the APL-derived NB4 cells in soft agar (33). Cells transfected with expression vector containing *PML* showed more than 50% reduction in colonies formed on soft agar. Cells transfected with control plasmid (pSG5) and *PML* mutants (PSG5*PML*mut and pSG5*PML*/RARα) did not show inhibition of colony growth. Furthermore, we also show that *PML* suppresses the transformation of REF and NIH3T3 cells by oncogenes. In all of these experiments the fusion product *PML*/RARα fails to suppress the tumorigenic growth of NB4 cells as well as the transformation of the REF and NIH3T3 cells. These results suggest that the translocation of APL inactivated the biological function of *PML* as a tumor suppressor and that this molecular alteration may be a precipitating event in the development of APL.

We have examined the transactivation of the *p21*$^{WAF1/CIP1}$ promoter by PML/RARα. Our results showed that *p21*$^{WAF1/CIP1}$ is a target gene for PML/RARα. Deletion analysis revealed a PML/RARα response element in the *p21*$^{WAF1/CIP1}$ promoter. Furthermore, transient expression of *PML/RARα* was found to induce *p21*$^{WAF1/CIP1}$ expression. These results suggest that PML/RARα may regulate the proliferation and differentiation by activating the transcription of *p21*$^{WAF1/CIP1}$.

Body

In this grant period (August 1, 1997 - July 31, 1998), we determine whether PML/RARα could stimulate the transcription of the *p21*$^{WAF1/CIP1}$ gene, a construct containing 2.3 kb of the *p21*$^{WAF1/CIP1}$ promoter placed in front of a bacterial chloramphenicol acetyltransferase (CAT) reporter gene, was transiently cotransfected with the *PML/RARα* expression plasmid into human lung carcinoma H1299 cells. The expression of p21 is highly regulated by p53 (34). Therefore, the H1299 cells, which are p53-/-, were used in this study to eliminate any potential transactivation of the *p21*$^{WAF1/CIP1}$ promoter reporter by p53 that could interfere with the effect of the *PML/RARα*. Fig. 1A (**Please see attached manuscript for figures**) showed that the expression of PML/RARα in H1299 cells resulted in an induction of CAT activity in comparison with the pSG5 vector control. The induction was further increased to approximately 17-fold when the transfected cells were treated with RA for 24 h (Fig. 1A). The induction of the CAT activity by RA was mediated by PML/RARα, since addition of RA alone did not result in increased CAT activity (Fig. 1A). Consistent with other reports, our result also indicated that PML/RARα is a ligand-binding transcription factor (25-27). Comparable transactivation of the *p21*$^{WAF1/CIP1}$ promoter was also observed in other cell types including the human breast cancer SK-Br3 (Fig. 1B) and the cervical carcinoma HeLa cells (data not shown). These results indicated that the *p21*$^{WAF1/CIP1}$ gene is a target for PML/RARα and the activation is RA responsive.

We next examined whether it was the PML or the RARα moiety of the fusion protein that activated the *p21*$^{WAF1/CIP1}$ promoter activity. Moderate transactivation of the promoter was observed with PML in either H1299 or SK-Br3 transfected cells but no further alterations in CAT activity was observed in the presence of RA (Fig. 1). Similar transactivation was also observed in HeLa cells (data not shown). However, no significant activation of the *p21*$^{WAF1/CIP1}$ promoter was found with RARα, either in the presence or the absence of RA (Fig. 1). These results suggested that the activation of the *p21*$^{WAF1/CIP1}$ promoter by PML/RARα may not be due to the RARα domain. As controls, we also examined the effect of p53 on *p21*$^{WAF1/CIP1}$ promoter in H1299 and SK-Br3 cells. Expression of wild type p53 in these cells resulted in a strong induction

of the $p21^{WAF1/CIP1}$ promoter activity while a mutant p53 failed to transactivate it (Fig. 1).

Since PML/RARα can transactivate the $p21^{WAF1/CIP1}$ promoter, we performed deletion analysis to determine the putative PML/RARα response element in the $p21^{WAF1/CIP1}$ promoter using a series of deletion mutants cloned into a luciferase reporter vector (35) (Fig. 2). The deletion constructs were cotransfected with the $PML/RAR\alpha$ expression plasmid into H1299 cells either in the presence or absence of RA. As shown in Fig. 2, progressive deletion of the $p21^{WAF1/CIP1}$ promoter up to nucleotide -94, relative to the transcription start site, conferred response to activation by PML/RARα and this was significantly increased in the presence of RA. However, deletion of the promoter sequences downstream of -94 abolished PML/RARα basal promoter activity and transactivation by PML/RARα (Fig. 2). Further deletion up to -60 abrogated residual RA induced promoter activity through PML/RARα. These results suggest that the sequence between -94 and -60 is required for transactivation by PML/RARα in the presence of RA (Fig. 2). Since this region is also essential for basal promoter function, therefore, PML/RARα may interact with basal transcription factors to activate $p21^{WAF1/CIP1}$ gene transcription.

To further verify the function of this PML/RARα response element, we placed the promoter sequence between -94 and -65 containing the putative response element in an expression vector immediately upstream of a minimal HSV thymidine kinase (TK) promoter, which drives the expression of a CAT reporter gene (Fig. 3A). The PML/RARα response element conferred an approximately 4-fold increase in promoter activity and further induction of the promoter was observed in the presence of RA (Fig. 3B). These results suggest that the region between -94 and -65 of the $p21^{WAF1/CIP1}$ gene promoter can mediate the transactivation by PML/RARα and this activation can be further stimulated in the presence of RA.

To determine whether PML/RARα can bind to this putative response element, electrophoretic mobility shift assay (EMSA) was performed with synthetic oligonucleotides containing sequences between -94 and -61 of the $p21^{WAF1/CIP1}$ promoter using nuclear extracts from either NB4 or HL60 cells. The NB4 cell line is derived from an APL patient and expresses the PML/RARα fusion protein (36). The HL60 cells do not express the PML/RARα fusion protein. Comparable patterns of band shift were noted for both NB4 and HL60 nuclear extracts and the shifted complexes can be competed with an excess of unlabeled PML/RARα response element oligonucleotides (Fig. 4A), but not those containing an irrelevant p53 response element (data not shown). Since the region between -94 and -61 also contains Sp1 elements (37-39), addition of polyclonal antibody against Sp1 caused supershifting of one of the major protein-DNA complexes, thus confirming the presence of Sp1 factor in NB4 and HL60 extracts (Fig. 4B). In contrast, an antibody against PML produced a supershifted band in NB4 but not the HL60 extracts (Fig. 4B), suggesting the presence of PML/RARα in the complex.

To investigate whether $p21^{WAF1/CIP1}$ expression can be induced by PML/RARα in cultured cells, we transiently transfected H1299 cells with the PML/RARα expression plasmid. Immunohistochemical staining by an anti-p21$^{WAF1/CIP1}$ antibody showed the presence of p21$^{WAF1/CIP1}$ in PML/RARα transfected cells (Fig. 5B). No staining was detected in vector transfected cells (Fig. 5A). In addition, cells transfected with a wild-type p53 expression plasmid exhibited significant increase in $p21^{WAF1/CIP1}$ expression (Fig. 5C). These results demonstrated that PML/RARα can induce $p21^{WAF1/CIP1}$ gene expression in cultured cells.

Conclusions

The universal cell cycle inhibitor p21$^{WAF1/CIP1}$ was first identified as a target gene for the tumor suppressor p53 (34). In this study, we demonstrated that PML/RARα can transactivate from the p21$^{WAF1/CIP1}$ promoter in an RA-dependent manner. Deletion analysis revealed a region in the promoter between -94 and -66 relative from the transcription start site that is required for

transactivation by PML/RARα. When this region was fused to a heterologous HSV TK minimal promoter, it can confer PML/RARα stimulation, with further response to RA. Therefore, genetic alterations of *PML* including gene mutations, and aberrant chromosomal translocation such as those in acute promyelogenous leukemia involving RARα, may be common in breast cancer. Indeed expression of *PML* has been shown to be altered in breast cancer (40). In normal breast specimens, less than 3% of the epithelial cells exhibit *PML* staining, but increasing levels of *PML* was detected as the lesions progress from benign dysplasias to carcinomas. *PML* expression is also markedly reduced when malignant cells become invasive (34). These studies further suggest that *PML* may play a critical role in breast carcinogenesis. We reasoned that aberrant regulation of p21 by *PML* may contribute to the development of breast cancer. Future studies will involve the characterization of the regulatory relationship between *PML* and p21.

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Transcriptional Activation of the Cyclin-Dependent Kinase Inhibitor p21 by PML/RARα

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Abstract

Acute promyelocytic leukemia (APL) is a result of clonal expansion of hematopoietic precursors blocked at the promyelocytic stage and is associated with a t(15;17) chromosomal translocation and the expression of the PML/RARα fusion protein. Treatment of APL cells with retinoic acid (RA) leads to complete remission by inducing growth arrest and differentiation of these cells into granulocytes. The cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ may be involved in terminal differentiation associated growth arrest. We showed in this study that PML/RARα increased the transcription of *p21$^{WAF1/CIP1}$* gene and the activation was further induced by RA treatment. Deletion analysis revealed a region upstream of the *p21$^{WAF1/CIP1}$* promoter that is required for transactivation by PML/RARα. Transient transfection of PML/RARα in cells increased the endogenous p21$^{WAF1/CIP1}$ protein levels. These results suggest that the induction of APL cells differentiation by RA may be a result of the activation of *p21$^{WAF1/CIP1}$* by PML/RARα.

1. Introduction

Acute promyelocytic leukemia (APL) is characterized by clonal proliferation and expansion of hematologic precursors at the promyelocytic stage of myeloid differentiation. The hallmark t(15;17) translocation associated with APL results in the fusion of the *PML* gene on chromosome 15 with the retinoic acid receptor α (*RARα*) gene on chromosome 17, generating the PML/RARα fusion protein [1-3]. PML/RARα retains most of the functional domains of PML and RARα. The PML moiety of the fusion protein contains the RING domain which may be involved in DNA binding or transcription regulation, and a coiled-coil region that mediates the formation of dimerization with PML [4-8]. The RARα moiety retains the DNA and RA binding domains and the interface for dimerization with RXR [4,7,8]. The physiological function of PML is unclear. It has been shown that PML may suppress growth and transformation [9,10]. RARα, on the other hand, is a transcription factor and a member of the nuclear hormone receptor superfamily, and is involved in regulating development and differentiation [11,12]. The PML/RARα fusion protein can either transcriptionally activate or repress RA-response gene in a ligand-dependent manner [4,7,13]. Therefore, PML/RARα may have dominant negative controls over the RARα or PML pathways [7,10,13,14].

Interestingly, APL patients can be treated with RA to induce terminal differentiation of APL cells toward mature granulocytes and achieve transient complete remission [15-17]. The mechanisms of this RA induced differentiation is still unclear. Induction of terminal differentiation is a complex process and may involve the regulation of a cadre of genes including those directly responsible for cell cycle withdrawal and growth arrest. The regulation of the cyclin-dependent kinase (CDK) inhibitor p21$^{WAF1/CIP1}$ has been shown to play an important role in terminal differentiation associated growth arrest in several experimental systems [18,19] and in differentiating tissues *in vivo* [20,21].

In this study, we have examined the transactivation of the *p21$^{WAF1/CIP1}$* promoter by PML/RARα. Our results showed that *p21$^{WAF1/CIP1}$* is a target gene for PML/RARα. Deletion

analysis revealed a PML/RARα response element in the *p21*$^{WAF1/CIP1}$ promoter. Furthermore, transient expression of *PML/RARα* was found to induce *p21*$^{WAF1/CIP1}$ expression. These results suggest that PML/RARα may regulate the proliferation and differentiation of the APL cells by activating the transcription of *p21*$^{WAF1/CIP1}$.

2. Materials and methods

2.1. Cell lines

H1299 lung carcinoma, SK-Br3 breast carcinoma, HeLa cervical epithelioid carcinoma, HL60 and NB4 promyelocytic leukemia cells were maintained in either DMEM or RPMI 1640 media (Life Technologies, Inc.) containing 10% fetal bovine serum (Gemini Bio-products, Inc.). All media were supplemented with L-glutamine, penicillin, and streptomycin.

2.2 . Plasmid Constructs

The CAT reporter plasmid containing 2.3 kb of human *p21*$^{WAF1/CIP1}$ promoter sequences and the various *p53* constructs were provided by Dr. A. Levine (Princeton University, Princeton, NJ). The cloning of *PML, RARα,* and *PML/RARα* expression plasmids were as described [9]. The *p21*$^{WAF1/CIP1}$ promoter deletion mutants were cloned into a luciferase reporter as described [22]. The plasmid containing the p21$^{WAF1/CIP1}$ promoter sequences between -94 and -65 was obtained by subcloning a *Hind*III-*Sal*I fragment (-94 to +16) from the -94 deletion mutant [22] into the *Hind*III-*Sal*I digested pBLCAT5 [23]. After transformation, the plasmid was digested by *Apa* I and *Xba* I, blunt-ended, and ligated.

2.3. Transient-transfection Assays

H1299, HeLa and SK-Br3 cells were tansfected with various plasmid constructs as indicated by calcium phosphate-DNA coprecipitation [24]. Approximately 16 h following transfection, the media were refreshed and 1 μM of RA or its solvent (DMSO) was added. Cells were then harvested 24 h later for the appropriate reporter assays. Luciferase activity was measured using the luciferase assay reagent from Promega (Madison, WI) according to the

specification of the manufacturer. CAT and luciferase activities were normalized based on protein concentrations.

2.4. Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from HL60 and NB4 cells as described [25]. Oligonucleotides corresponding to the *p21*$^{WAF1/CIP1}$ promoter sequences between -94 and -61 were synthesized, annealed and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled oligonucleotides were incubated with 10 µg of nuclear extract in a buffer containing 20 mM Hepes (pH 7.9), 60 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 250 µg/ml bovine serum albumin, 2 µg of poly (dA-dT), and 12% glycerol at room temperature for 15 min. Competition experiments were performed by adding unlabeled oligonucleotides, with either the same sequence or an unrelated sequence (p53 response element), to the binding reactions 15 min prior to the addition of the labeled oligonucleotide. For supershift experiments, antisera against either Sp1 (Santa Cruz Biotechnology) or PML (a gift from Dr. K.S. Chang, M.D. Anderson Cancer Center, Houston, TX) were added to the binding reaction for 1 h at 4°C, prior to the addition of radiolabeled oligonucleotide. Binding reactions were resolved on a 4% nondenaturing polyacrylamide gel at 200 volts for 2 h at 4°C in 0.5X TBE buffer. The gels were subsequently dried and autoradiography was performed.

2.5. Immunohistochemistry

H1299 cells were grown on chamber slides and transfected with 3 µg of either the pSG5 control vector, the *PML/RARα* expression plasmid or the *p53* expression plasmid using Lipofectamine (Gibco/BRL). Approximately 40 h after transfection, cells were washed in Hank's balanced salt solution (Gibco/BRL) and fixed for 10 min with 95% ethanol/5% acetic acid at -20°C. Fixed cells were incubated in PBS at room temperature for 10 min, then with 1% hydrogen peroxidase for 15 min, 2% normal horse serum in PBS for 1 h, and with a 1:20 dilution of the mouse anti-p21$^{WAF1/CIP1}$ antibody (Ab-1, Oncogene) at 4°C overnight. Bound antibody was detected by using Vectastain ABC peroxidase kits (Vector Laboratories, Burlingame, CA)

according to the manufacturer's specification. Peroxidase was performed with 3, 3'-Diaminobenzidine as a chromogenic substrate.

3. Results

3.1. Transcriptional activation of p21$^{WAF1/CIP1}$ by PML/RARα

To determine whether PML/RARα could stimulate the transcription of the $p21^{WAF1/CIP1}$ gene, a construct containing 2.3 kb of the $p21^{WAF1/CIP1}$ promoter placed in front of a bacterial chloramphenicol acetyltransferase (CAT) reporter gene, was transiently cotransfected with the *PML/RARα* expression plasmid into human lung carcinoma H1299 cells. The expression of p21 is highly regulated by p53 [26]. Therefore, the H1299 cells, which are p53$^{-/-}$, were used in this study to eliminate any potential transactivation of the $p21^{WAF1/CIP1}$ promoter reporter by p53 that could interfere with the effect of the *PML/RARα*. Fig. 1A showed that the expression of PML/RARα in H1299 cells resulted in an induction of CAT activity in comparison with the pSG5 vector control. The induction was further increased to approximately 17-fold when the transfected cells were treated with RA for 24 h (Fig. 1A). The induction of the CAT activity by RA was mediated by PML/RARα, since addition of RA alone did not result in increased CAT activity (Fig. 1A). Consistent with other reports, our result also indicated that PML/RARα is a ligand-binding transcription factor [4,7,13]. Comparable transactivation of the $p21^{WAF1/CIP1}$ promoter was also observed in other cell types including the human breast cancer SK-Br3 (Fig. 1B) and the cervical carcinoma HeLa cells (data not shown). These results indicated that the $p21^{WAF1/CIP1}$ gene is a target for PML/RARα and the activation is RA responsive.

We next examined whether it was the PML or the RARα moiety of the fusion protein that activated the $p21^{WAF1/CIP1}$ promoter activity. Moderate transactivation of the promoter was observed with PML in either H1299 or SK-Br3 transfected cells but no further alterations in CAT activity was observed in the presence of RA (Fig. 1). Similar transactivation was also observed in

HeLa cells (data not shown). However, no significant activation of the *p21$^{WAF1/CIP1}$* promoter was found with RARα, either in the presence or the absence of RA (Fig. 1). These results suggested that the activation of the *p21$^{WAF1/CIP1}$* promoter by PML/RARα may not be due to the RARα domain. As controls, we also examined the effect of p53 on *p21$^{WAF1/CIP1}$* promoter in H1299 and SK-Br3 cells. Expression of wild type p53 in these cells resulted in a strong induction of the *p21$^{WAF1/CIP1}$* promoter activity while a mutant p53 failed to transactivate it (Fig. 1).

3.2. Localization of PML/RARα response element in the p21$^{WAF1/CIP1}$ promoter

Since PML/RARα can transactivate the *p21$^{WAF1/CIP1}$* promoter, we performed deletion analysis to determine the putative PML/RARα response element in the *p21$^{WAF1/CIP1}$* promoter using a series of deletion mutants cloned into a luciferase reporter vector [22] (Fig. 2). The deletion constructs were cotransfected with the *PML/RARα* expression plasmid into H1299 cells either in the presence or absence of RA. As shown in Fig. 2, progressive deletion of the *p21$^{WAF1/CIP1}$* promoter up to nucleotide -94, relative to the transcription start site, conferred response to activation by PML/RARα and this was significantly increased in the presence of RA. However, deletion of the promoter sequences downstream of -94 abolished PML/RARα basal promoter activity and transactivation by PML/RARα (Fig. 2). Further deletion up to -60 abrogated residual RA induced promoter activity through PML/RARα. These results suggest that the sequence between -94 and -60 is required for transactivation by PML/RARα in the presence of RA (Fig. 2). Since this region is also essential for basal promoter function, therefore, PML/RARα may interact with basal transcription factors to activate *p21$^{WAF1/CIP1}$* gene transcription.

To further verify the function of this PML/RARα response element, we placed the promoter sequence between -94 and -65 containing the putative response element in an expression vector immediately upstream of a minimal HSV thymidine kinase (TK) promoter, which drives the expression of a CAT reporter gene (Fig. 3A). The PML/RARα response element conferred an approximately 4-fold increase in promoter activity and further induction of the promoter was

observed in the presence of RA (Fig. 3B). These results suggest that the region between -94 and -65 of the $p21^{WAF1/CIP1}$ gene promoter can mediate the transactivation by PML/RARα and this activation can be further stimulated in the presence of RA.

3.3. Electrophoretic mobility shift analysis of the PML/RARα response element

To determine whether PML/RARα can bind to this putative response element, electrophoretic mobility shift assay (EMSA) was performed with synthetic oligonucleotides containing sequences between -94 and -61 of the $p21^{WAF1/CIP1}$ promoter using nuclear extracts from either NB4 or HL60 cells. The NB4 cell line is derived from an APL patient and expresses the PML/RARα fusion protein [27]. The HL60 cells do not express the PML/RARα fusion protein. Comparable patterns of band shift were noted for both NB4 and HL60 nuclear extracts and the shifted complexes can be competed with an excess of unlabeled PML/RARα response element oligonucleotides (Fig. 4A), but not those containing an irrelevant p53 response element (data not shown). Since the region between -94 and -61 also contains Sp1 elements [28-30], addition of polyclonal antibody against Sp1 caused supershifting of one of the major protein-DNA complexes, thus confirming the presence of Sp1 factor in NB4 and HL60 extracts (Fig. 4B). In contrast, an antibody against PML produced a supershifted band in NB4 but not the HL60 extracts (Fig. 4B), suggesting the presence of PML/RARα in the complex.

3.4. Induction of $p21^{WAF1/CIP1}$ gene expression by PML/RARα

To investigate whether $p21^{WAF1/CIP1}$ expression can be induced by PML/RARα in cultured cells, we transiently transfected H1299 cells with the PML/RARα expression plasmid. Immunohistochemical staining by an anti-p21$^{WAF1/CIP1}$ antibody showed the presence of p21$^{WAF1/CIP1}$ in PML/RARα transfected cells (Fig. 5B). No staining was detected in vector transfected cells (Fig. 5A). In addition, cells transfected with a wild-type p53 expression plasmid exhibited significant increase in $p21^{WAF1/CIP1}$ expression (Fig. 5C). These results demonstrated that PML/RARα can induce $p21^{WAF1/CIP1}$ gene expression in cultured cells.

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4. Discussion

The universal cell cycle inhibitor p21$^{WAF1/CIP1}$ was first identified as a target gene for the tumor suppressor p53 [26]. In this study, we demonstrated that PML/RARα can transactivate the *p21*$^{WAF1/CIP1}$ promoter in a RA-dependent manner. Deletion analysis revealed a region in the promoter between -94 and -61 relative from the transcription start site that is required for transactivation by PML/RARα. When this region was fused to a heterologous HSV TK minimal promoter, it can confer PML/RARα stimulation with further response to RA. Gel shift assay indicated that the PML/RARα chimera may form a complex with an oligonucleotide derived from the promoter encompassing nucleotides between -94 and -61. Most importantly, transient transfection of PML/RARα induced expression of the endogenous p21$^{WAF1/CIP1}$ in cultured cells. The transcriptional activation of *p21*$^{WAF1/CIP1}$ by PML/RARα is p53-independent because H1299 cells are p53$^{-/-}$. Furthermore, deletion of the p53 response element in the *p21*$^{WAF1/CIP1}$ promoter did not affect the stimulation of the promoter activity by PML/RARα (Fig. 2). Therefore, *p21*$^{WAF1/CIP1}$ is a physiological target gene for PML/RARα.

In this study, we identified a PML/RARα response element in the *p21*$^{WAF1/CIP1}$ promoter located between -94 and -60. The region between -1212 and -1194 in the promoter has previously been shown to contain the retinoic acid response element (RARE) [31]. In contrast, the *PML/RARα* response element between -94 and -61 in the *p21*$^{WAF1/CIP1}$ promoter does not contain any consensus for retinoic acid. Our results thus suggest that the activation of *p21*$^{WAF1/CIP1}$ transcription in H1299 or SkBr-3 cells may not be mediated by the RARα domain. Therefore, the domain of PML/RARα that binds the promoter may be due to the PML moiety of the fusion protein. Indeed, we observed a moderate increase in the transcription of *p21*$^{WAF1/CIP1}$ by cotransfection with PML but not with RARα. In addition, we also did not observe further activation of the *p21*$^{WAF1/CIP1}$ promoter in the presence of RA, or cotransfecting with an RARα

expression plasmid in these cell types. Since RARα binding to RARE may require heterodimerization with RXR [8,31], therefore, the failure to respond to RA stimulation may be due to a lack of expression of RXR in these cells. The absence of RXR may result in the preferential formation of PML/RARα homodimers, instead of PML/RARα/RXR heterodimers, whose DNA binding pattern is distinct from those of PML/RARα homodimeric complexes [8].

Activation of the $p21^{WAF1/CIP1}$ promoter by PML/RARα occurs in a region that overlaps with the TGFβ response element mapped in HaCaT cells [28], the okadaic acid response element in U937 cells [29], the butyrate response element in human colon cancer cell line [30], and the region required for induction during mouse keratinocyte differentiation [32]. This region of the promoter is also required for basal promoter activity. Therefore, the interaction of PML/RARα with $p21^{WAF1/CIP1}$ promoter could be through its association with other nuclear factors. The presence of Sp1/Sp3 consensus site in this region has been confirmed and shown to bind Sp1 and Sp3 factors [28-30,32]. Antibodies against either Sp1 or PML produce supershifts in one of the DNA-protein complexes (Fig.4), indicating that both Sp1 and PML/RARα can bind the putative PML/RARα response element. We have shown recently that PML can interact with Sp1 (S. Vallian, K.-V. Chin and K.-S. Chang, unpublished data), therefore it is conceivable that PML/RARα may interact with Sp1 to regulate $p21^{WAF1/CIP1}$ transcription by protein-protein interactions through the PML domain. Alternatively, it is also possible that PML/RARα can directly bind to the consensus in the promoter.

It is increasingly clear that CDK inhibitors including p21$^{WAF1/CIP1}$ may be involved in cell growth and differentiation. For example, myoD upregulates $p21^{WAF1/CIP1}$ expression, which is correlated with muscle cell differentiation [21]. Agents that induce myeloid cell differentiation have also been shown to activate $p21^{WAF1/CIP1}$ expression [29,33,34]. Paradoxically, p21$^{WAF1/CIP1}$ at low concentrations can promote the association of cyclin and CDK subunits to initiate cell cycle progression [35]. It has been shown that cells treated with growth factor was accompanied by low level induction of $p21^{WAF1/CIP1}$ [36]. Therefore, we suggest that this aberrant activation of

$p21^{WAF1/CIP1}$ by the PML/RARα chimera may be the underlying pathogenic mechanism of APL and its retinoid response. In the absence of RA, PML/RARα may induce low levels of $p21^{WAF1/CIP1}$ expression and result in the promotion of proliferation and transformation of myeloid cells. In contrast, RA treatment of leukemic cells further enhances $p21^{WAF1/CIP1}$ expression by PML/RARα, leading to growth arrest, leukemic maturation, and clinical remission of APL patients. Based on this hypothesis, we predict that agents that raise $p21^{WAF1/CIP1}$ expression may induce differentiation of APL cells. Indeed, a recent study has revealed that 12-O-tetradecanoyl-phorbol 13-acetate (TPA), which induces $p21^{WAF1/CIP1}$ expression [29,33,34], can stop the proliferation of the APL patient derived NB4 cells [37]. Associated with this proliferation arrest was cell differentiation along myeloid cell lineages [37]. Thus, induction of terminal differentiation of leukemic cells by physiological or pharmacological modulators may control the growth of the malignant cells and has therapeutic implications.

In summary, our studies have revealed that the PML/RARα fusion protein may act as a double-edged sword. Its activation of the $p21^{WAF1/CIP1}$ gene paradoxically drives the transformation and clonal proliferation of myeloid cells, yet also induces terminal differentiation of APL cells toward mature granulocytes and complete remission after treatment with RA. These results may explain the pathogenesis of APL. Therefore, pharmacological modulation of $p21^{WAF1/CIP1}$ expression may be beneficial for the treatment of APL.

Acknowledgments

Footnote

Abbreviations: APL, acute promyelocytic leukemia; RA, retinoic acid; EMSA, electrophoretic mobility shift assay; RARE, retinoic acid response element.

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Figure Legends

Fig. 1. Trancsription activation of p21$^{WAF1/CIP1}$ by PML/RARα. H1299 (A) and SK-Br3 (B) cells were cotransfected with p21-CAT (4 μg) and the indicated expression plasmids (4 μg) in the presence or the absence of 1 μM RA. The amount of DNA in each cotransfection was kept constant through the addition of ssDNA to 10 μg. RA was added 16 h after transfection. The cells were harvested 40 h after transfection and assayed for CAT enzymatic activities which were normalized for cellular protein concentrations.

Fig. 2. Deletion analysis of the p21$^{WAF1/CIP1}$ promoter. p21$^{WAF1/CIP1}$ full-length and deletion promoter reporter constructs (5 μg) were cotransfected with 5 μg of the PML/RARα expression plasmid or ssDNA into H1299 cells in the presence or the absence of 1 μM RA. RA was added 16 h after transfection. The cells were harvested 40 h after transfection and assayed for luciferase activities which were normalized for cellular protein concentration. TATA represents the p21$^{WAF1/CIP1}$ TATA box located 45 bp from the transcription start site (defined as +1). The 5'-boundaries of the reporters are indicated to the left of each construct and all the constructs shown share the same 3'-boundary located at +16 bp downstream of the p21$^{WAF1/CIP1}$ transcription-initiation site. S1and S2 indicate p53 binding sites. Shown is a representive experiment done in three times.

Fig. 3. Stimulation of transcription via PML/RARα response element in the p21$^{WAF1/CIP1}$ promoter. A, the p21$^{WAF1/CIP1}$ promoter sequence between -94 and -65 was fused to pBLCAT5. TK*p*, thymidine kinase promoter from herpes simplex virus gene; CAT, gene encoding CAT. B, the pBLCAT5 and pPRRE (the construct in A) were cotransfected with the PML/RARα expression plasmid or the control vector pSG5 into H1299 cells in the presence or the absence of 1μM RA. The CAT activity was measured and normalized for cellular protien concentration. Fold induction was determined by comparing normalized CAT activity in cells transfected with the PML/RARα expression plasmid to cells transfected with the control vector pSG5.

Fig. 4. Gel mobility shift analyses of PML/RARα binding to the p21$^{WAF1/CIP1}$ promoter. A, nuclear extract (10 μg) from HL60 (lane 2) or NB4 cells (lane 3) were incubated with an end-labeled duplex DNA probe corresponding to regions -94 to -61 of the p21$^{WAF1/CIP1}$ promoter sequence and competed with either a 20-fold (lane 4 and 5) or a 400-fold (lane 6 and 7) molar excess of the unlabeled specific competitor. B, nuclear extract (10 μg) from HL60 cells or NB4 cells were incubated with the labeled DNA probe and anti-Sp1 antibody (lane 4-6), or anti-PML antibody (lane 7-9). P, free probe without adding nuclear extract; H, nuclear extract from HL60 cells; N, nuclear extract from NB4 cells. The arrow indicates the complex supershifted with anti-PML antibody.

Fig. 5. Induction of the expressioin of p21$^{WAF1/CIP1}$ protein by transient transfection with PML/RARα into H1299 cells. H1299 cells were transfected with the pSG5 vector (A), PML/RARα (B), or p53 (C) expression plasmid and stained with anti-p21 antibody. Arrows indicate cells stained with anti-p21$^{WAF1/CIP1}$ antibody.
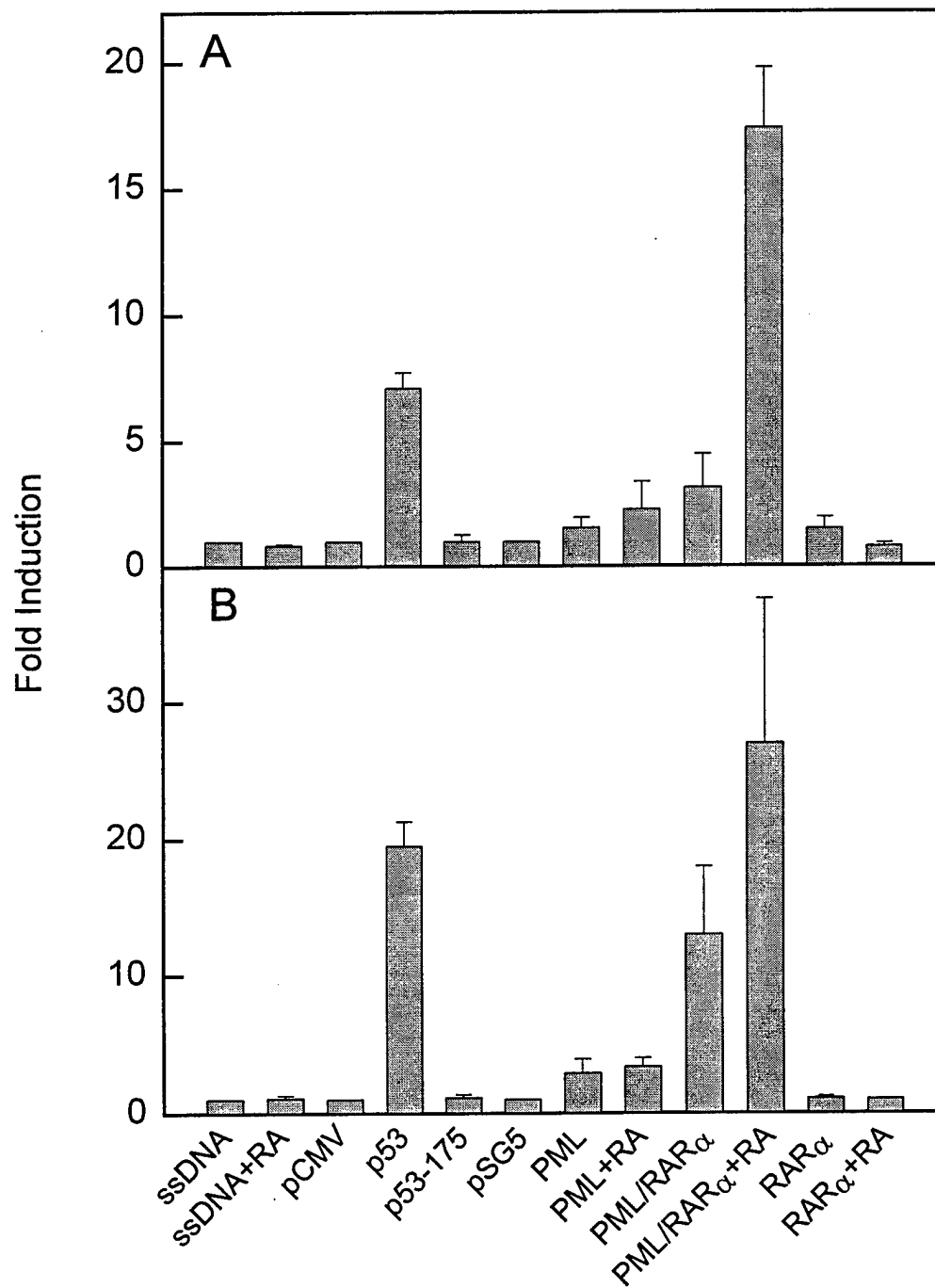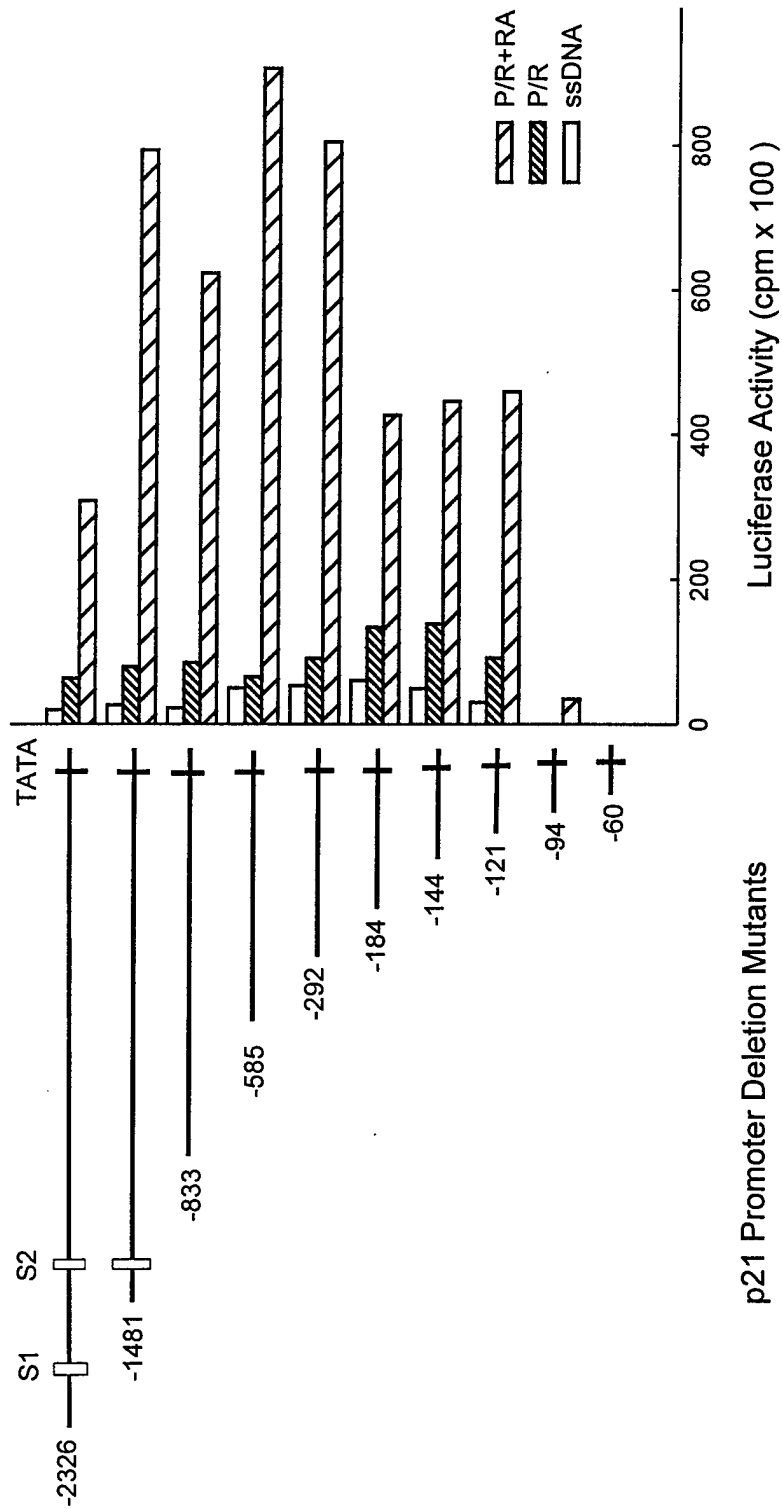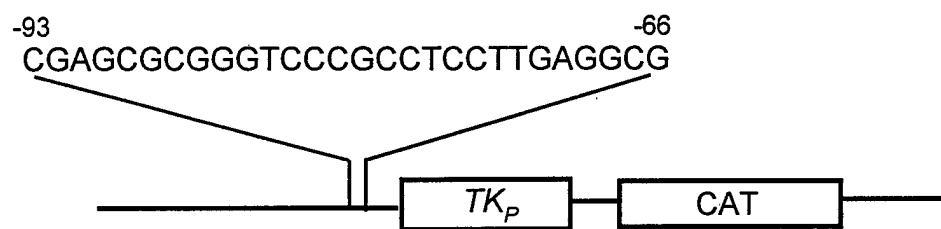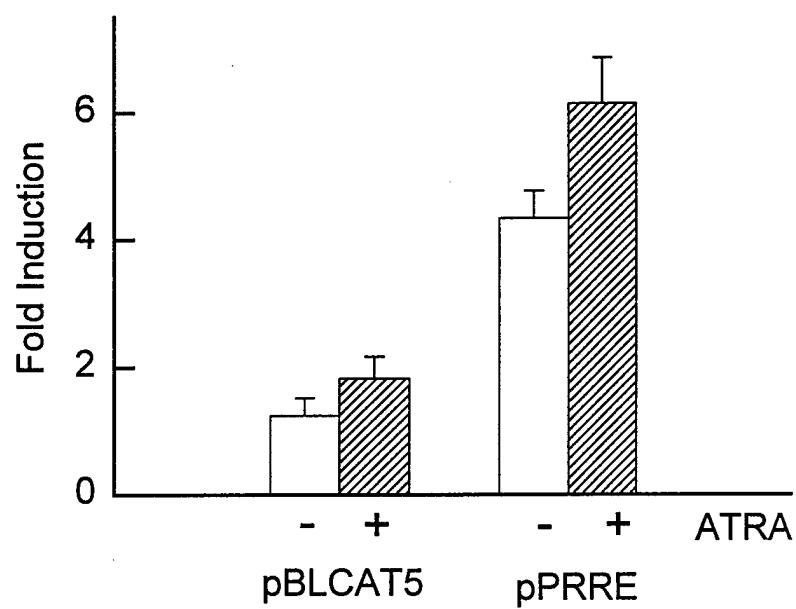
Figure 1

Figure 2

P/R+RA

P/R

ssDNA

Luciferase Activity (cpm x 100)

p21 Promoter Deletion Mutants

TATA

S1 S2

-2326

-1481

-833

-585

-292

-184

-144

-121

-94

-60

Figure 3

A

$$-93 \qquad\qquad\qquad\qquad\qquad -66$$

CGAGCGCGGGTCCCGCCTCCTTGAGGCG

TK$_P$ CAT

B

Figure 4

Figure 5

A

B

C